Effect of Local Puerarin Administration on Xenograft

Lokal Puerarin Uygulamasının Hayvansal Kemik Grefti Üzerine Etkisi

Effect of Puerarin on Ossification

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Özet

Amaç: Bu çalışmanın amacı, ratlarda oluşturulan kritik boyutta kalvaryal kemik defektlerinde, lokal olarak uygulanan Puerarin'in hayvansal kemik grefti üzerine etkisini incelemektir. Gereç ve Yöntem: Yirmidört adet rat kalvaryasında 5 mm çapında kritik boyutta kemik defekti oluşturuldu. Denekler Grup C (pasif kontrol), Grup XBG (Bio-oss uygulanan grup), Grup P-XBG (puerarin+Bio-oss uygulanan grup). Bütün deneklere operasyondan 28 gün sonra ötenazi işlemi uygulandı. Yeni kemik ve bağ dokusu hacmi ölçümü için stereolojik analizler kullanıldı. Bulgular: P-XBG, XBG ve C gruplarının ortalama kemik hacimleri sırasıyla, 1,57 ± 0.13mm3, 1.24 ± 0.10 mm3 and 0,31 ± 0.09 mm3 olarak belirlendi. Yeni kemik hacmi değerleri incelendiğinde P-XBG ve XBG gruplarında yeni kemik hacmi, kontrol grubuna göre istatistiksel olarak anlamlı bulundu ($p \leq 0.05$). Ayrıca P-XBG ve XBG grupları karşılaştırıldığında, P-XBG grubundaki yeni kemik hacmi XBG grubuna göre istatistiksel olarak daha fazla bulundu ($p \leq 0.05$). P-XBG grubunda ortalama olarak 2.00 ± 0.12 mm3 bağ doku hacmi ölçüldü. P-XBG ve XBG gruplarındaki bağ dokusu hacmi kontrol grubuna göre anlamlı derecede fazla bulunurken, P-XBG ve XBG grupları karşılaştırıldığında ise iki grup arasındaki fark yine istatistiksel olarak anlamlı bulundu ($p \leq 0.05$). Tartışma: Çalışma sonucunda puerarinin Bio-oss üzerinde pozitif bir etki yarattığı ve yeni kemik oluşumunu arttırdığı belirlenmiştir.

Anahtar Kelimeler

Ksenogreft; Kemik Oluşumu; Kalvarya

Abstract

Aim: The purpose of this study was to investigate the healing potential of local puerarin administration on xenograft in cranial bone defect treatment in rats. Material and Method: A critical-size defect of 5-mm diameter was created in the calvarium of twenty-four rats. The animals were divided into three groups: group C (passive control), group XBG (treated with Bio-Oss), and group P-XBG (treated with puerarin + Bio-Oss combination). All animals were euthanized at 28 days postoperative. Stereologic analyses were performed. New bone area and connective tissue volumes were measured. Results: Mean new bone volumes were 1.57 ± 0.13mm3, 1.24 ± 0.10 mm3, and 0.31 ± 0.09 mm3 in groups P-XBG, XBG and C respectively. The new bone volumes were statistically significant in groups P-XBG and XBG compared to group C ($p \leq 0.05$). Moreover, the new bone formation in group P-XBG was statistically higher when compared to the XBG group ($P<0.05$). In the P-XBG group, mean connective tissue volume was 2.00 ± 0.12 mm3. The connective tissue volumes were statistically higher in groups P-XBG and XBG compared to group C ($p<0.05$). The difference between group P-XBG and group XBG was also statistically significant ($p<0.05$). Discussion: Puerarin had a positive effect on xenograft and increased new bone formation.

Keywords

Xenograft; Bone Formation; Calvaria

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Introduction

Bone remodeling is comprised of two phases: bone formation and bone resorption through the activity of osteoclasts, osteoblasts, and osteocytes. The balance between the two phases is crucial for the maintenance and regeneration of alveolar bone [1]. In the oral cavity, bone defects can be seen as small dental defects and as major defects spreading to the jaws. Autografts or xenografts have been used to reconstruct bone defects. Autografts, considered the gold standard, have the highest osteogenic capability with sufficient mechanical strength and do not induce immune responses. However, the amount of autologous bone available for grafting is limited and there is a requirement for a second surgical site [2]. Xenografts are bone grafts taken from a donor of a different type. This natural material that is similar to human bone shows great osteoconductive activity and is commonly used in dentistry [3].

Phytoestrogens are plant compounds similar to mammalian estrogen and its metabolites, found in a wide variety of foods [4]. Pueraria, the root of Pueraria lobata is a commonly used phytoestrogen. The major active ingredient of pueraria is called isoflavone, which has five basic types. Puerarin is one of the isoflavones widely used in alternative medicine. A growing body of work reports that puerarin has been widely used in the treatment of diabetes and diabetic complications, osteonecrosis, Parkinson's disease, and cardiovascular and cerebrovascular diseases [4]. Puerarin has a wide spectrum of pharmacological uses, such as in promoting bone formation, vasodilation, antioxidation, cardioprotection, and anti-inflammation [5]. In addition, according to a recent study, puerarin can promote osteoblast formation in-vitro [6].

The aim of the present study was to evaluate the effect of locally administered puerarin with xenograft on cranial bone defects in rats using stereological analysis methods.

Material and Method

Experimental Model

Twenty-four Sprague-Dawley (SD) rats weighing 200 to 250 g were randomly divided into three groups: group C, group XBG, and group P-XBG. This study was approved by the Animal Experimentation Committee of Bülent Ecevit University, Zonguldak, Turkey.

Surgical Procedures

After anesthesia by intramuscular injection of 3 mg/kg xylazine hydrochloride (Rompuns; Bayer, Leverkusen, Germany) and 35 mg/kg ketamine hydrochloride (10% Ketasol; Richter Pharma AG, Wels, Austria) per kilogram, the surgical procedures were performed. The hair over the calvarium was removed. The cutaneous surface was disinfected with povidone iodine solution. A semilunar incision was made and the full thickness flaps were retracted. A 5mm defect was created with a trephine using a low-speed hand-piece. During preparation of the defect, sterile saline was used for irrigation to reduce thermal injury to the bone. Care was also taken in all animals to avoid damaging the dura mater.

In group C, defects were left unfilled and were allowed to heal spontaneously without using any grafting material. In group XBG, defects were filled with only Bio-Oss® (Bio-Oss, Geistlich Biomaterials, Wolhusen, Switzerland). In group P-XBG, defects were filled with a combination of Bio-Oss® (Bio-Oss, Geistlich Biomaterials, Wolhusen, Switzerland) and 20 mg puerarin. Puerarin (99%) (Sigma-Aldrich, USA), soluted with distilled water (50mg/ml), was prepared fresh on the day of the operation for the puerarin-used group. The flaps were sutured after operation with resorbable 4/0 polyglactin 910 sutures.

For postoperative infection control and analgesia, each animal was injected with both 10 mg cefazolin sodium (Sefazol; M Nevzat, Istanbul, Turkey) per kilogram and 200 mg metamizol sodium (Novalgin; Aventis, Istanbul, Turkey) for 5 days after the operation. The animals were euthanized by overdose anesthesia 4 weeks after the operation. The calvarias were removed from the scalp, cleaned, and placed in 10% tempered formaldehyde solution.

Histological and Stereological Analysis

After the decapitation of the rats, the calvarial samples were decalcified in formic acid (5%) for 21 days, then soaked in neutral 10% formaldehyde, dehydrated in a graded alcohol series, and cleared in xylol for light microscopy examination. After the preparation of the paraffin blocks, 7 µm-thick sections were taken. For volume estimation, each 20th section was sampled in a systematic random sampling manner and stained with hematoxylin and eosin.

The sections selected and stained with hematoxylin and eosin were photographed using a stereology analysis system (Stereoinvestigator 9.0; Microbrightfield, Williston, VT, USA) and a light microscope (M4000 B; Leica Instruments) equipped with a digital color camera (Microbrightfield). The unbiased Cavalieri method was applied to the light microscopy images to stereologically estimate the volume of new bone using point-counting test grids. The point density of the point-counting grids was designed to obtain an appropriate coefficient of error (CE) for the area of interest in the images of the serial sections [7]. The grid, with its systematic array of points, was placed randomly on the image shown on the screen of a personal computer (Figure 1). The volume of each area of interest in each section was estimated with the following formula:

Volume = $t \times a/p \times \sum p$,

where t is the section thickness, a/p is the area of each point on the point-counting grid, and $\sum p$ is the total number of points within the area of interest. The CE and coefficient of variation were estimated according to the formula of Gundersen and Jensen [7].

Statistical Analysis

Statistical analysis was performed using a commercially available software program (SPSS version 19.0; SPSS Inc., Chicago, IL, USA). The Shapiro–Wilk test was used to confirm whether the data were normally distributed. The stereological parameters were analyzed using the Kruskal-Wallis nonparametric test, followed by post-hoc group comparisons with the Bonferroni-adjusted Mann–Whitney U test, after normality assumption of data had been rejected ($P<0.05$). (The Bonferroni correction, $\alpha=0.05/3=0.016$, was applied to determine statistical significance). $P < 0.05$ was considered to indicate statistical significance.

Results

There was no mortality detected and no wound infections observed throughout the experimental period.

Histological Evaluation

Control Group; Histological analysis showed bone formation in all rats. New bone formation was close to the defects borders. Almost all defect area was filled by a thin connective tissue layer.

XBG Group; Bone formation was found to be greater than that in the control group, with a larger amount of newly-formed bone tissue. Almost all defects were occupied by remnants of the bone graft particles. Most bone graft particles showed empty osteocyte lacunae.

P-XBG Group; Histological analysis showed bone formation in all rats. Most bone graft particles were surrounded by newly formed bone. Large areas of intense resorption of the bone graft particles were observed in most of these specimens (Figure 2).

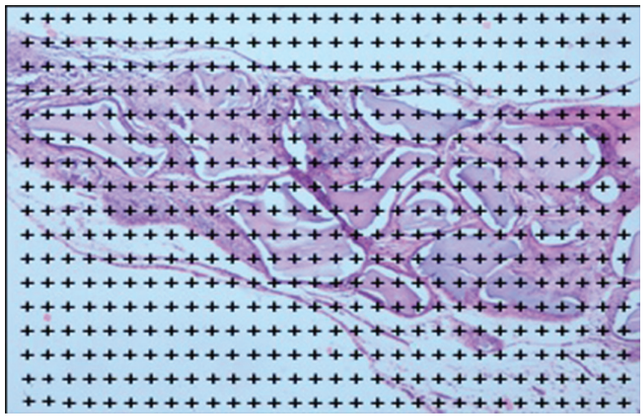

Figure 1. Photo of stereological evaluation with counting grid.

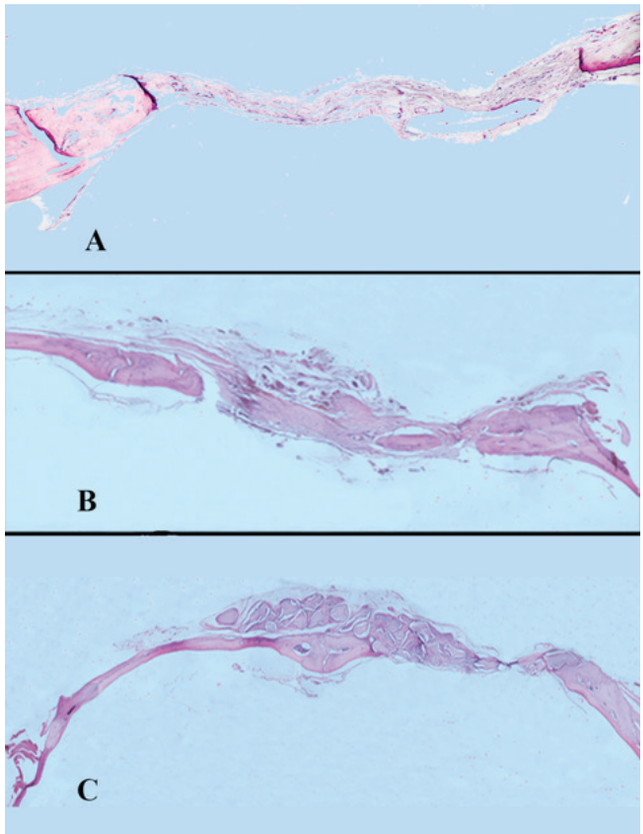

Figure 2. Panaromic view of defect area. Group C (a), group XBG (b), group P-XBG (c)

Stereological Analysis

Mean bone volume in group P-XBG was 1.57 ± 0.13mm3, whereas mean volumes in the XBG and C groups were 1.24 ± 0.10 mm3 and 0.31 ± 0.09 mm3 respectively (Figure 3). The new bone volumes were statistically significant in groups P-XBG and XBG when compared to group C ($p < 0.05$). Moreover, bone formation in group P-XBG was statistically higher than that in group XBG ($p < 0.05$).

The connective tissue volumes data are shown in Figure 4. In group P-XBG, mean volume was 2.00 ± 0.12 mm3, whereas mean volume values in the XBG and C groups were 1.75 ± 0.15 mm3 and 0.97 ± 0.14 mm3, respectively. The connective tissue volumes were statistically higher in groups P-XBG and XBG compared to group C ($p < 0.05$). The difference between group P-XBG and group XBG was also statistically significant ($p < 0.05$).

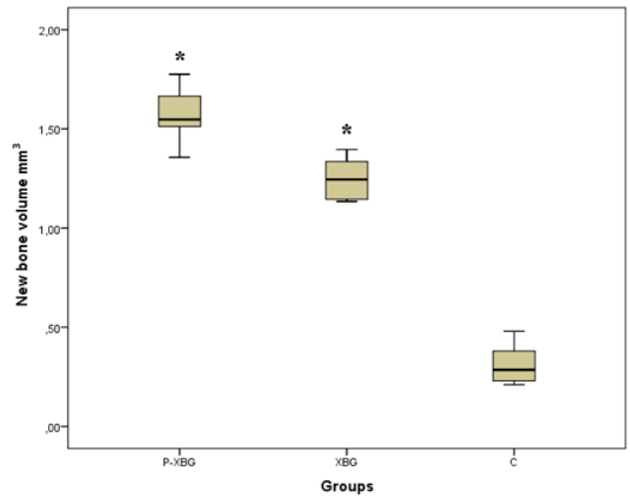

Figure 3. New bone volume data in study groups.
* Statistically significant difference among groups (Kruskal-Wallis/Bonferroni-adjusted Mann–Whitney U)

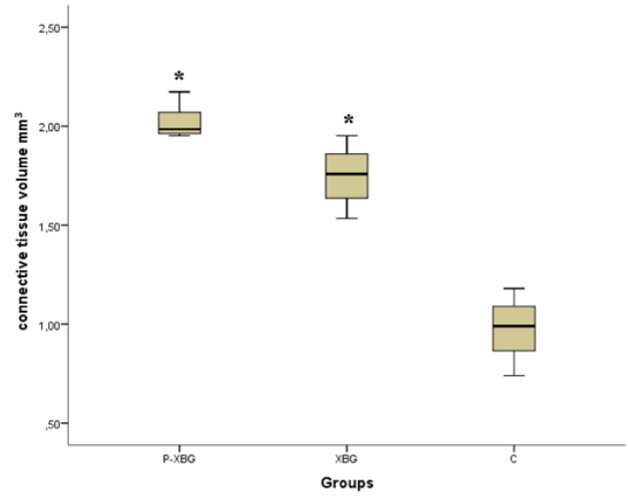

Figure 4. Connective tissue volume data in study groups
* Statistically significant difference among groups (Kruskal-Wallis/Bonferroni-adjusted Mann–Whitney U)

Discussion

The objective of this study was to evaluate the new bone formation volume of cranial defects in rats treated with xenograft and puerarin + xenograft combination. Consistent with the study hypothesis, stereological measures showed superior bone regeneration within the puerarin + xenograft treated defects compared to the defects that were untreated or treated solely with Bio-Oss.

Autograft is still considered the gold standard for bone regeneration; it has osteoconductive and osteoinductive effects without any associated immune response [8]. On the other hand, autograft has disadvantages such as second surgical site requirement and limited supply of bone sources [2]. Alternatively there are various bone graft materials that have been studied on their own or in combination: xenografts, allografts, and synthetic grafts [9-11]. Despite disadvantages, such as causing an immune response by the host and slow degradation, xenografts have been used successfully in numerous studies, including guided bone regeneration (GBR) [12] and sinus augmentation [13]. In addition, the studies conducted on rat calvarial defects with Bio-Oss have also shown positive results on bone formation [14,15]. Therefore, in this research, as commonly used in dentistry, a natural bone mineral of bovine origin (BioOss, Geistlich, Switzerland) was used as the xenograft.

Oral bone defects can be seen as a result of systemic diseases and also from periodontal and peri-implant diseases, trauma, tumor or congenital defects [16]. The present study tests the hypothesis that puerarin can enhance the effect of xenograft on bone regeneration.

The rat calvarial bone defect model is a quick, frequently used model for in-vivo evaluation of bone healing. The advantages of this model on bone regeneration are easy manipulation and no requirement for wound stabilization [17,18]. Due to the similarity between compressive force exerted on the rat calvaria and the one being used in treatment of intraoral wounds, the rat calvaria bone defect model is preferred [17].

Critical-size defects are defined as bone defects that do not heal spontaneously during the animal's lifetime [19]. In rats, calvarial defects 5 mm in diameter are regarded as critical-sized defects [20]. In this study the critical-sized bone defect of the rat calvaria was used to evaluate bone regeneration.

There are not many studies evaluating the effects of local puerarin application on rats, so in the present study the puerarin dose was determined by considering a previous study that evaluated the bone formation effect of locally administered puerarin in bone defect [21]. Based on this information, the puerarin dose selected and administered was 20 mg per defect.

Using only histological examinations alone may not be sufficient to demonstrate the newly formed bone volume. Sterio (1984) described several modifications of the approaches used to estimate the volume of objects in 3D space [22]. The validity has been well established [23]. Therefore, in this study the combined effects of graft material and puerarin extract on bone regeneration were evaluated using stereologic analyses. To our knowledge, no previously published study has described the effect of puerarin on xenograft material to induce bone regeneration using stereological analysis.

As mentioned, an isoflavone extracted from the root of the Pueraria Lobata plant, Puerarin, is an important extract used for bone-related diseases like osteoporosis [5]. The effect of puerarin and its role in preventing osteoporosis has been discussed in the literature recently and its antiosteoporotic effects were found [24]. There is data about puerarin acting as a growth stimulator for osteoblasts [25] and as exerting effects that promote proliferation, differentiation, and mineralization. While it showed positive effects on the development of osteoblasts in low dose, high-dose puerarin could inhibit the formation of bone [6]. Based on these reports, we tested the hypothesis that puerarin would have positive effects on xenograft in bone regeneration in rats.

During the histological evaluation in this study, it was observed that new bone formation was evident in all groups. In the control group, new bone formation was close to the borders of the defects and almost all of the defect area was filled by a thin connective tissue layer. In the XBG and P-XBG groups almost all defects were occupied by remnants of the bone graft particles. Additionally, it was found that the group in which the puerarin + xenograft combination was used showed the best healing results histologically, and can be used as an alternative. To the best of the authors' knowledge, puerarin had been used in this way only in a single study in which the critical-size bone defects were created in a rabbit model for bone regeneration. Authors evaluated the new bone by comparing one group with puerarin in collagen matrix and one group with collagen matrix alone [21]. They found that there was more new bone formation at the host bone-graft interface in the puerarin + collagen matrix group compared to the group in which the collagen matrix was used alone.

In our study, stereological analyses also showed accordance with our histological evaluations. The highest new bone volume was found in the P-XBG group. In the XBG group our results showed superior new bone volume compared to the control group. Moreover, there was a statistically significant difference between the P-XBG and XBG groups. To our knowledge, this is the first stereological study evaluating the effects of the administration of puerarin alone and puerarin together with Bio-Oss® bone graft material on bone regeneration.

In summary, according to our histological and stereological findings, this experimental study showed that puerarin could have positive effects on xenograft compared with control and xenograft groups. Further studies are needed for establishing the optimal dose to maximize the anabolic actions and to minimize the side effects of puerarin on bone. Within the limitations of the study, puerarin could be seen as a potential material to stimulate bone formation from graft material.

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Competing interests

The authors declare that they have no competing interests.

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